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Regulation of Fat Cell Mass by Insulin in *Drosophila melanogaster*▽

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A phylogenetically conserved response to nutritional abundance is an increase in insulin signaling, which initiates a set of biological responses dependent on the species. Consequences of augmented insulin signaling include developmental progression, cell and organ growth, and the storage of carbohydrates and lipids. Here, we address the evolutionary origins of insulin's positive effects on anabolic lipid metabolism by selectively modulating insulin signaling in the fat body of the fruit fly, *Drosophila melanogaster*. Analogous to the actions of insulin in higher vertebrates, those in *Drosophila* include expansion of the insect fat cell mass both by increasing the adipocyte number and by promoting lipid accumulation. The ability of insulin to accomplish the former depends on its capacity to bring about phosphorylation and inhibition of the transcription factor *Drosophila* FOXO (dFOXO) and the serine/threonine protein kinase shaggy, the fly ortholog of glycogen synthase kinase 3 (GSK3). Increasing the amount of triglyceride per cell also depends on the phosphorylation of shaggy but is independent of dFOXO. Thus, the findings of this study provide evidence that the control of fat mass by insulin is a conserved process and place dFOXO and shaggy/GSK3 downstream of the insulin receptor in controlling adipocyte cell number and triglyceride storage, respectively.

The recent epidemic of obesity, insulin resistance, and type 2 diabetes mellitus (T2DM) has renewed interest in fundamental principles of metabolic homeostasis. Much attention has been directed to the appropriate control of carbohydrate metabolism, as elevated glucose is the cardinal, defining sign of diabetes mellitus. Nonetheless, an integrated and finely tuned balance of lipid storage and breakdown is also essential for metabolic well-being. Defects in lipid metabolism not only contribute directly to a number of diseases, such as the inherited lipodystrophies, the metabolic syndrome, and atherosclerosis, but also are an integral component of T2DM. In fact, it has been argued persuasively that T2DM is regarded as a disorder of glucose metabolism for purely historical reasons and that lipid abnormalities are as or more important to the pathogenesis and consequences of the disease (33). Thus, a refined, mechanistic understanding of the normal control of lipid storage and mobilization is critical.

The major regulators of lipid synthesis and breakdown are glucose and the peptide hormone insulin. It is now clear that insulin signals through a canonical pathway that is phylogenetically conserved, controlling growth, development, and ageing in invertebrates (*Caenorhabditis elegans* and *Drosophila melanogaster*) and metabolic outputs in vertebrates (rodents and humans) (17, 37). For example, in mammals, insulin regulates hepatic, muscle, and adipocyte glucose metabolism in a manner dependent on signaling through phosphatidylinositol 3-kinase (PI3K) and its downstream intermediary, Akt, also known as protein kinase B (12, 44). This same pathway has also been implicated in insulin's action in the central nervous system to modulate carbohydrate metabolism and energy balance (39). Much less clarity exists concerning the relevant insulin-signaling pathway(s) in the regulation of lipid metabolism, particularly that occurring in liver. Although there is consensus that insulin promotes triglyceride synthesis in liver by augmenting the expression and activity of the lipogenic transcription factor sterol response element binding protein 1c (SREBP1c), data regarding additional relevant signaling pathways have been conflicting (46). For example, there is support for either atypical protein kinase C or Akt as the major transducer of prolipogenic signals, and both FoxO1 and FoxA2 have been suggested as major alternative relevant transcription factors in addition to SREBP1c (31, 32, 50, 57, 59). Even more so than hepatocytes, adipocytes are capable of varying their levels of stored neutral lipid over a wide range by altering synthesis, accumulation, oxidation, and mobilization. The total fat mass contained within adipose tissue is also subject to regulation of the number of fat cells. Whereas there is substantial understanding of the signaling pathways governing differentiation into fat cells, the key factors determining the number of cells representing the precursor stem cell pool are not known. Nonetheless, insulin and/or insulin-like growth factor 1 is an important extracellular agent influencing adipogenesis (41).

The utility of *D. melanogaster* as a relevant model system with which to investigate and understand components of human biology, including the insulin-signaling pathway, is now well established (3, 45). Using the fly, considerable recent effort has been directed toward both defining the physiological role of insulin and delineating its downstream signaling pathways that control cell size and number as well as longevity (17, 51). A model has emerged in which insulin serves as a phylogenetically conserved nutritional sensor, in invertebrates controlling such primitive functions as growth and development but in vertebrates having evolved and broadened to be the prime regulator of anabolic functions, such as the synthesis and redistribution of simple nutrients into long-term energy stores. It is also likely that insulin exerts influence on the control of *Drosophila* metabolism (3). For example, flies deficient in insulin-like peptides display increases in extracellular reducing sugars that can be abolished by ectopic expression of an insu-

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lin-like peptide gene (43). However, less attention has been given to the roles of these same molecules in lipid metabolism. Flies with a loss of insulin-like peptide-producing cells or mutations in the gene for *Drosophila* insulin receptor (dInR) or the scaffold protein chico (the *Drosophila* ortholog of insulin receptor substrate) have a significant increase in triglyceride (4, 8, 52). While reminiscent of the human obesity that predisposes to insulin-resistant states, an increase in total triglyceride is the opposite of the typical consequence of insulin-deficient states in mice and humans (44). This has called into question the relevance of *Drosophila* as a model for mammalian lipid metabolism. Nonetheless, a number of proteins and pathways integral to the control of lipid metabolism in higher organisms have homologs in flies, in some cases with demonstrably comparable functions. For example, the *Drosophila* genome encodes both a lipid binding protein and a triglyceride lipase quite similar to proteins expressed in human adipocytes and critical to hormone-regulated lipolysis (20, 21). Moreover, these enzymes appear to participate in the control of lipid storage in flies in a manner analogous to that in mammals (22). Like the human liver, the *Drosophila* fat body packages lipids into lipoprotein particles for transport to peripheral tissues (10). Thus, lipid metabolism in insects may be more similar to that in mammals than once imagined. It is possible that the accumulation of lipid in flies deficient in insulin signaling may be misleading, possibly as a result of nonautonomous effects on energy metabolism. Consistent with this idea, larval fat body clones overexpressing dInR or the catalytic subunit of PI3K (Dp110) appear to accumulate more fat than controls, although this has been ascertained only indirectly by light or electron microscopy (7). In mice, deletion of the gene encoding insulin receptor substrate 2, which is homologous to chico, results in increased fat mass; this effect is thought to be secondary to a nonautonomous effect of hypothalamic insulin signaling on energy balance (9).

In this study, we address the issue of control of lipid metabolism in *Drosophila*, specifically considering the role of the insulin-signaling pathway in the fly's main nutrient storage organ, the fat body. We find that in flies, as in mammals, insulin is a positive regulator of fat cell mass, acting through changes in both cell number and lipid storage. We further go on to utilize *Drosophila* genetics to dissect downstream signaling and show distinct pathways by which insulin regulates the number of fat cells versus their triglyceride content.

MATERIALS AND METHODS

Fly genetics. The following fly strains were used in this study: UAS-dInR^{A1325D} (Bloomington 8263), UAS-GFP (Bloomington 5194), yolk-Gal4 (18), to-Gal4 (14), UAS-dTOR^{TED} (24), UAS-dFOXO-TM (27), UAS-sggS9A (5), and UAS-RBF (15).

Flies were grown on standard cornmeal-dextrose medium (128.4 g dextrose, 9.3 g agar, 61.2 g cornmeal, and 32.4 g Lake States dried *Torula* type b yeast per liter) supplemented with dry yeast (Sigma, St. Louis, MO), and crosses were performed at 25°C. To control for effects of the yolk-Gal4/takeout (to)-Gal4 driver, flies carrying each driver expressing green fluorescent protein (GFP) were compared to those carrying the driver expressing the given experimental transgene. To control for the genetic background and effects of the transgene alone, yolk-Gal4/FM7 or to-Gal4/CyO virgin females were crossed with males carrying the given experimental transgene and the resulting progeny carrying the driver and transgene were compared to their siblings carrying the transgene alone. To control for the genetic background in experiments including flies with more than one transgene, yolk-Gal4/FM7; sternopleural/+ virgin females were crossed with UAS-RBF, UAS-dTOR^{TED}, UAS-dFOXO-TM, or UAS-sggS9A males. Yolk-Gal4/y; sternopleural/transgene males were then crossed with UAS-dInR^{A1325D} virgin females, and the resulting siblings were analyzed. Control animals were cultured in the same vials as the experimental animals to normalize for larval crowding.

Protein, triglyceride, and DNA assays. Adult virgin female flies aged 4 to 5 days, males aged 7 to 10 days, or fat body-enriched preparations of tissues dissected from these animals, including the fat body cells that remained attached to the cuticles, were homogenized in lysis buffer containing 140 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% Triton X, and 1× protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were measured using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL), triglyceride concentrations were measured using the triglyceride LiquiColor kit (Stanbio Laboratory, Boerne, TX), and the total DNA content was measured using the Quant-iT double-stranded DNA high-sensitivity assay kit (Molecular Probes, Eugene, OR) according to the manufacturers' instructions. Each experiment was performed at least three times, and error bars in the figures represent standard errors of the means (SEM).

Nile Red staining and cell counting. For Nile Red staining, a 10% stock solution of Nile Red (Sigma-Aldrich, St. Louis, MO) in dimethyl sulfoxide was diluted 1:5,000 in a mixture of 1× phosphate-buffered saline, 30% glycerol, and 2 μg/ml DAPI (4′,6-diamidino-2-phenylindole; Roche Molecular Biochemicals, Indianapolis, IN). Four to six adult virgin female flies aged 4 to 5 days were dissected into this working solution, and cuticles with fat body cells attached were mounted whole and visualized using a Leica DM IRE2 confocal microscope. For each genotype, three to seven images of multiple fat bodies throughout the slide were taken randomly, and quantitation was performed with each image by using ImageJ 1.38x software (National Institutes of Health). For cell counting experiments, fat body cells attached to the cuticle were labeled with GFP genetically by expressing GFP under the control of the yolk-Gal4 driver and the cells were dissociated from one another and the cuticle in 0.5% trypsin-EDTA for 4 h with rocking. After 4 h, almost all of the dissociated cells were GFP positive. The majority of the GFP signal was found in the dissociated cells, with very little GFP found attached to the cuticle, suggesting that most of the fat body cells had been dissociated from one another and the cuticle at this time point. Cell numbers were determined by counting the total number of dissociated cells with a hemocytometer.

Antibodies. The phospho-*Drosophila* Akt (phospho-dAkt) and total dAkt antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA), and used at a 1:1,000 dilution. The E7 beta-tubulin antibody from Michael Klymkowsky was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, Iowa City. Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoblot analysis. Four- to five-day-old virgin female flies were dissected into phosphate-buffered saline, and fat body-enriched preparations of the cuticles with the fat body cells that remained attached were homogenized in lysis buffer containing 2% sodium dodecyl sulfate, 60 mM Tris-HCl, pH 6.8, 1× protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and 1× phosphatase inhibitor cocktail 1 (Sigma, St. Louis, MO). The lysates were then centrifuged for 15 min at 13,000 rpm and 4°C. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Whatman, Florham Park, NJ). Detection was performed using ECL reagents according to the instructions supplied by the manufacturer (Amersham Pharmacia Biotech). Each experiment was performed at least three times, and a representative blot is shown (see Fig. 4).

Statistical analysis. Unpaired Student's *t* tests were used for statistical analysis.

RESULTS

Insulin signaling promotes triglyceride storage in the *Drosophila* fat body. In order to understand the role of insulin in regulating triglyceride storage, we took a tissue-specific approach to manipulate insulin signaling in the *Drosophila* fat body, the major triglyceride storage organ of the fly. The insect fat body is an organ functionally analogous to the mammalian liver and adipose tissue and is present during both the larval and adult stages of development. The larval fat body stores

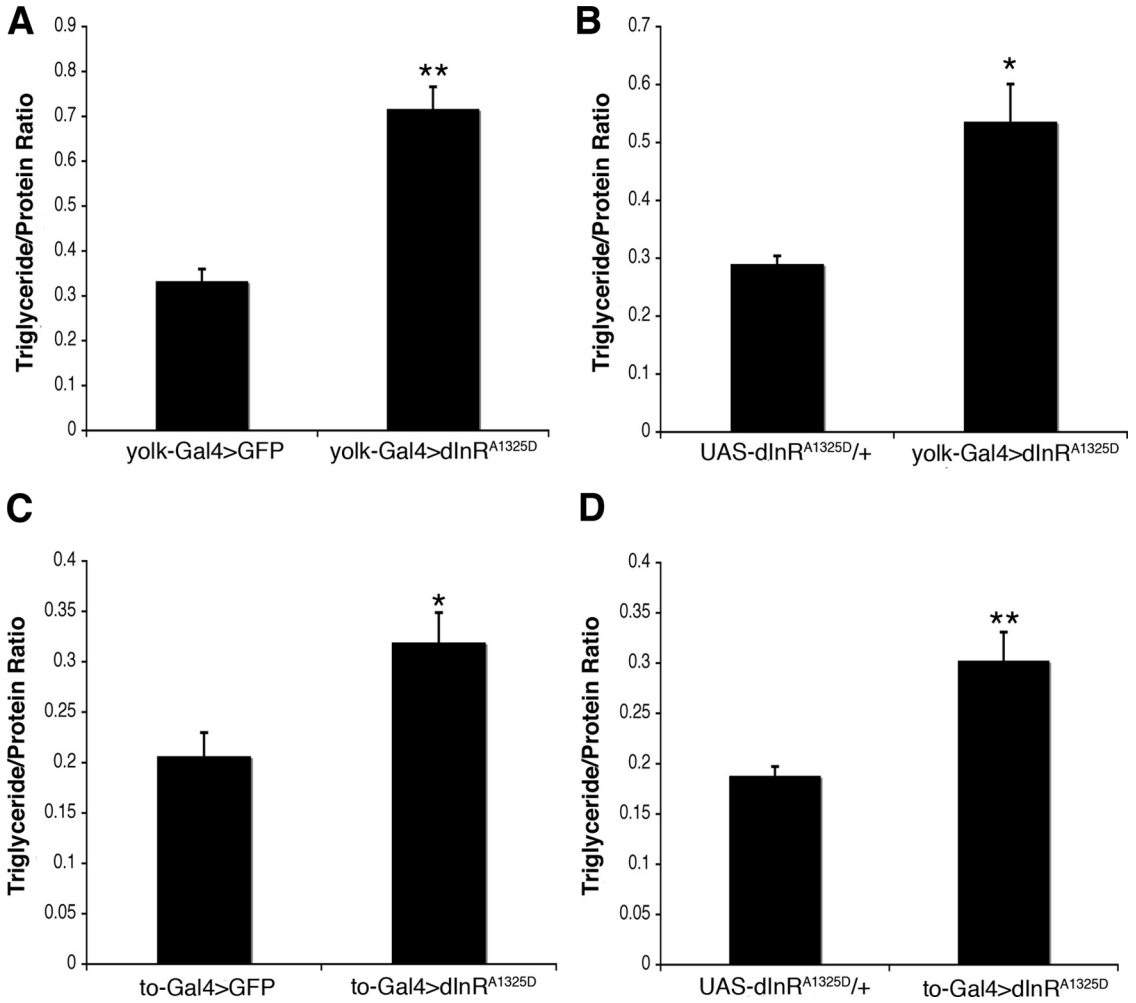

FIG. 1. Insulin signaling regulates lipid storage in the adult fat body. (A and B) Triglyceride/protein ratios in yolk-Gal4>dInR^{A1325D} 4- to 5-day-old adult virgin females compared to those in yolk-Gal4>GFP (A) and UAS-dInR^{A1325D}/+ (B) controls. (C and D) Triglyceride/protein ratios in to-Gal4>dInR^{A1325D} 7- to 10-day-old adult males compared to those in yolk-Gal4>GFP (C) and UAS-dInR$^{A1325D/+}$ (D) controls. Each experiment was performed at least three times; values are means ± SEM. *, $P < 0.05$, and **, $P < 0.01$ by unpaired Student's *t* test.

nutrients as glycogen and triglyceride and, during pupation, breaks down to supply the animal with enough energy to undergo metamorphosis (25, 36). The adult fat body is derived from a separate cell lineage from the larval fat body, and its cells are not easily identified until 3 to 4 days after eclosion (1, 26). To turn on insulin signaling in this tissue, we expressed an activated form of the insulin receptor, dInR^{A1325D}, in the female and male fat bodies by using the Gal4-upstream activation sequence (UAS) system with the yolk-Gal4 and to-Gal4 drivers, respectively. These drivers are expressed at high levels specifically in both the head and abdominal fat bodies by day 3, with lower-level expression starting at the end of pupal development for yolk-Gal4 and starting at eclosion for to-Gal4 (14, 18; J. R. DiAngelo and M. J. Birnbaum, unpublished observations). Expression of dInR^{A1325D} in the female fat body increased triglycerides (Fig. 1A and B). A similar phenotype was observed when dInR^{A1325D} was expressed in the male fat body (Fig. 1C and D). Together, these results suggest that activating insulin signaling in the fat body augments triglyceride storage.

Insulin increases adult fat body cell number. In increasing total triglyceride, insulin signaling may be affecting the number of fat body cells, the amount of triglyceride in each cell, or both. If the increase in triglycerides from expressing dInR^{A1325D} in the fat body was due to more fat per cell, then this increase in fat may result from larger or more abundant lipid droplets. To test this possibility directly, fat bodies were stained with the neutral lipid stain Nile Red. Activating insulin signaling only slightly increased the cytoplasmic area occupied by lipid droplets (Fig. 2). This finding is consistent with the trend toward an increase in triglyceride/protein and triglyceride/DNA ratios in dInR^{A1325D}-expressing fat bodies (Fig. 3C and E) (see below). While these data suggest that insulin does promote lipid accumulation in each cell, the increase in droplet area appears to be insufficient to account for the entire expansion in lipid mass measured in the dInR^{A1325D}-expressing animals. To assess the effect of insulin signaling on fat cell number, we dissociated fat body tissue enzymatically and counted the cells. Activating insulin signaling in the fat body led to an

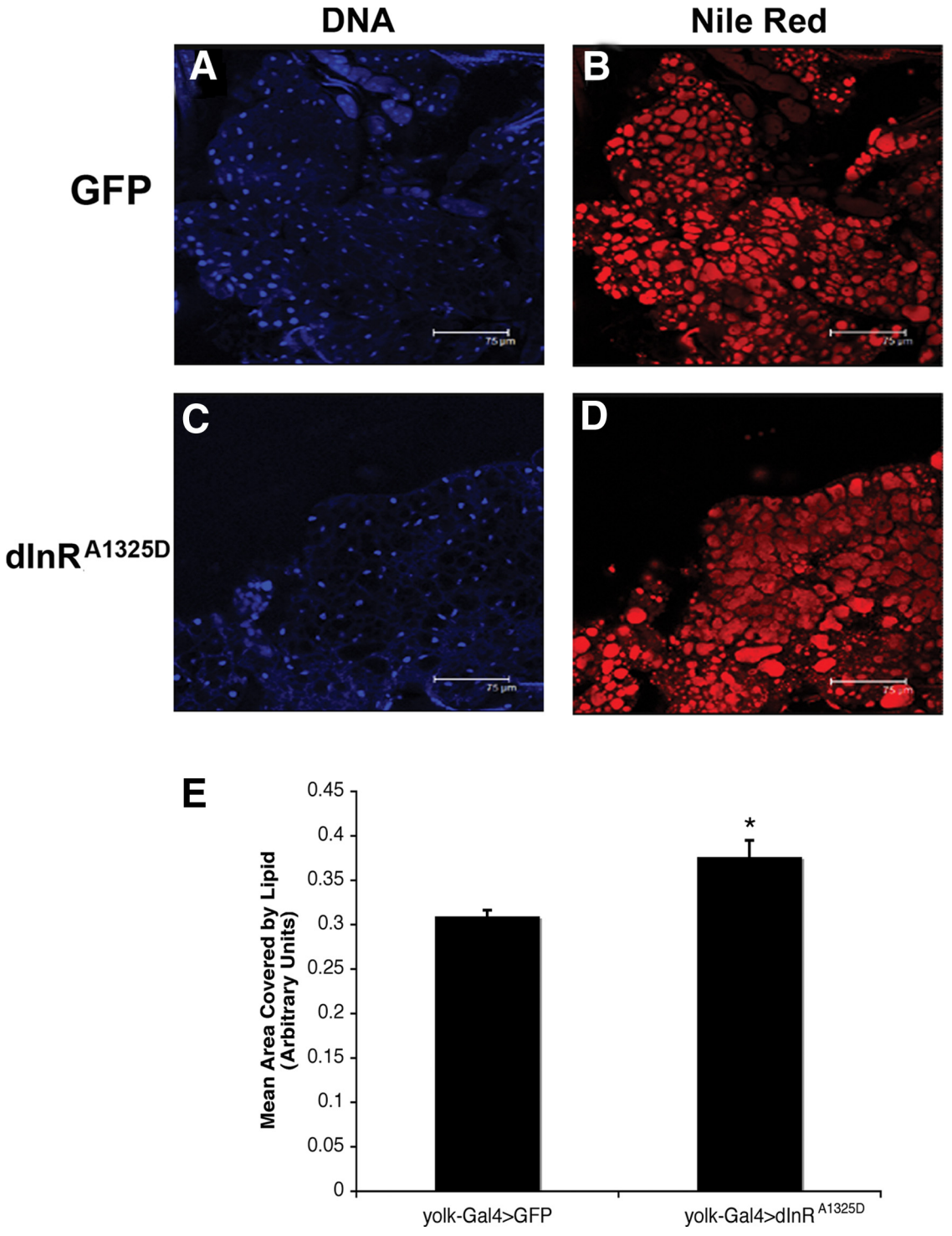

FIG. 2. Insulin increases the area covered by lipid droplets. (A to D) DAPI (A and C) and Nile Red (B and D) staining of isolated fat bodies from 4- to 5-day-old yolk-Gal4>GFP and yolk-Gal4>dInR^{A1325D} adult virgin females, respectively. Scale bars equal 75 μm. (E) Quantification of the mean area covered by lipid based on Nile Red staining of yolk-Gal4>GFP and yolk-Gal4>dInR^{A1325D} fat bodies is expressed in arbitrary relative units. Each experiment was performed at least three times, and values are means ± SEM. *, $P < 0.05$.

increase in the number of cells compared to tissues from the control animals (Fig. 3A). The total DNA content of dissected fat bodies reflected the number of cells, increasing to the same extent as the cell number in the dInR^{A1325D}-expressing fat body, and was therefore used as a surrogate measure of cell number (Fig. 3B and D). dInR^{A1325D} did not significantly increase the protein/DNA ratio in a normally proliferative fat body, consistent with there being no profound effect on fat cell

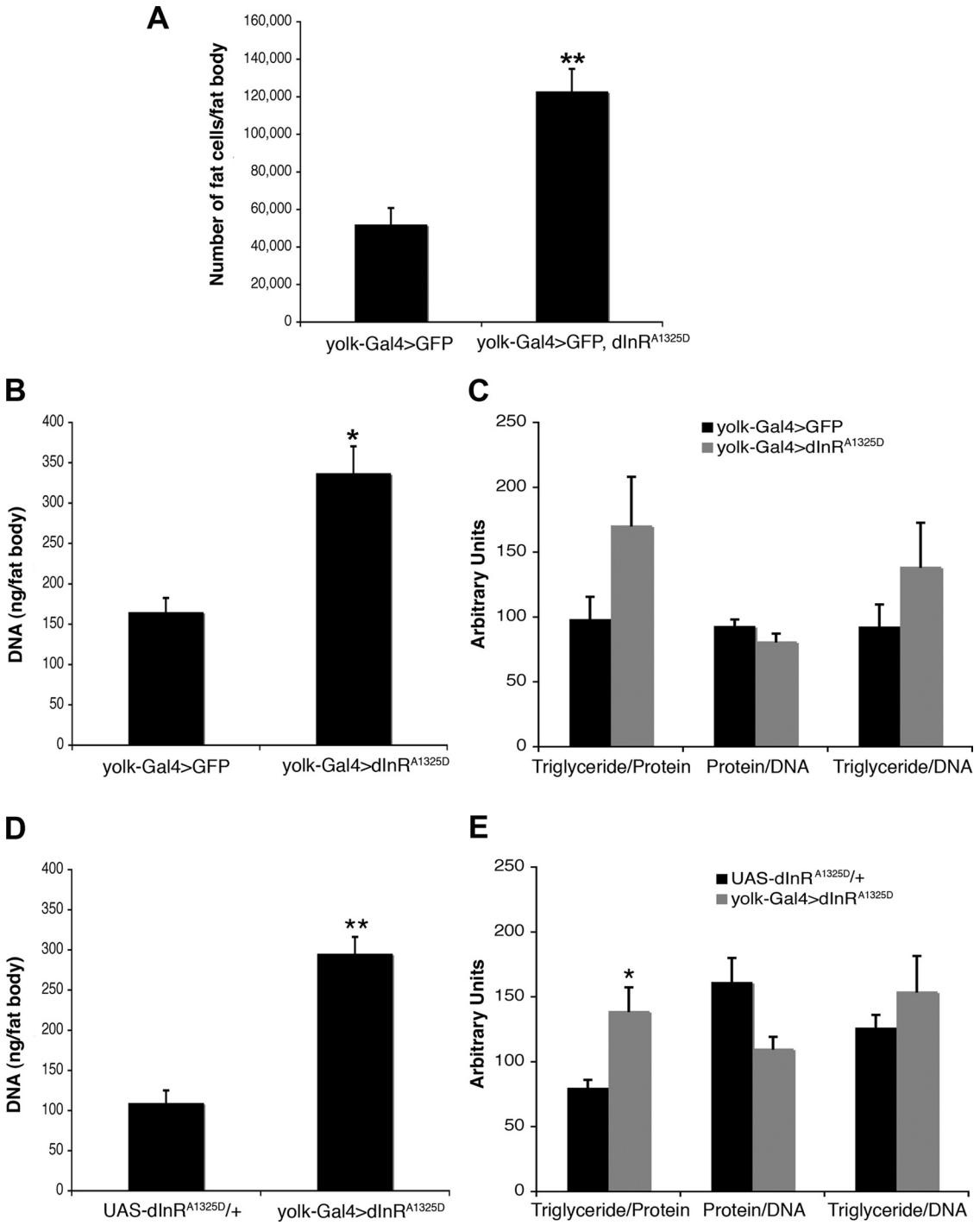

FIG. 3. Insulin signaling regulates fat cell number in the adult fat body. (A) Number of fat body cells dissociated after 4 h in 0.5% trypsin-EDTA from fat bodies dissected from 4- to 5-day-old adult yolk-Gal4>GFP, dInR^{A1325D} virgin females or yolk-Gal4>GFP control animals. (B) Total DNA content of fat bodies dissected from 4- to 5-day-old adult yolk-Gal4>dInR^{A1325D} virgin females or yolk-Gal4>GFP control animals. (C) Triglyceride/protein, protein/DNA, and triglyceride/DNA ratios in fat bodies dissected from yolk-Gal4>dInR^{A1325D} 4- to 5-day-old adult virgin females or yolk-Gal4>GFP control animals. (D) Total DNA content of fat bodies dissected from 4- to 5-day-old adult yolk-Gal4>dInR^{A1325D} virgin females or UAS-dInR$^{A1325D/+}$ control animals. (E) Triglyceride/protein, protein/DNA, and triglyceride/DNA ratios in fat bodies dissected from yolk-Gal4>dInR^{A1325D} 4- to 5-day-old adult virgin females or UAS-dInR^{A1325D}/+ control animals. Each experiment was performed at least three times, and values are means ± SEM. *, $P < 0.05$; **, $P < 0.01$.

size independent of triglyceride accumulation (Fig. 3C and E). As mentioned above, triglyceride/protein and triglyceride/DNA ratios tended toward an increase, again reflecting the modest increase in triglyceride per cell apparent from the light microscopy analysis (Fig. 2 and 3C and E). Overall, these data indicate that insulin positively regulates cell number in the adult fat body and that this regulation contributes significantly to the enhancement in triglyceride storage.

Insulin promotes triglyceride storage independently of changes in fat cell number. Since overexpressing dInR^{A1325D} in fat bodies revealed slightly larger lipid droplets than those in controls, we hypothesized that insulin signaling could regulate fat storage independently of changes in cell number. To test this hypothesis directly, we uncoupled proliferation from metabolism by holding the fat body cell number constant through inhibiting the cell cycle via overexpression of the *Drosophila* E2F corepressor retinoblastoma family homolog (RBF) (15). While RBF expression alone had no effect on the fat body DNA content or the triglyceride/protein, protein/DNA, or triglyceride/DNA ratio (Fig. 4A and B), RBF expression completely suppressed the increase in the fat body DNA content induced by dInR^{A1325D} (Fig. 3B and D and 4C). These data are most consistent with the idea that insulin signaling enhanced fat cell number by stimulating cell proliferation. Utilization of RBF also provided a strategy to assess the effect of insulin signaling on lipid levels independently of changes in fat cell number. To accomplish this assessment, we measured fat body triglycerides in animals that expressed both dInR^{A1325D} and RBF. These flies displayed increased triglyceride/protein ratios and, as there was no change in the DNA content, enhanced levels of triglyceride storage per cell (Fig. 4D). The ratio of protein to DNA was unchanged (Fig. 4D). These phenotypes were not caused by a reduction in insulin signaling due to the same driver's activating two effector lines at once, as levels of phospho-dAkt increased in animals expressing both dInR^{A1325D} and RBF compared to those in animals expressing RBF alone and the signal was comparable to that from driving of dInR^{A1325D} alone (Fig. 4E). These data indicate that insulin signaling exerts effects on lipid storage independently of the fat body cell number.

dFOXO and shaggy act downstream of dInR to regulate fat cell storage by different mechanisms. The insulin-signaling pathway regulates metabolism and growth by at least three distinct pathways downstream of dAkt: the *Drosophila* target of rapamycin (dTOR), shaggy (sgg)/glycogen synthase kinase 3 (GSK3), and transcription factor *Drosophila* FOXO (dFOXO) pathways (16, 17, 37, 38). In order to determine which of these are important to triglyceride accumulation, we performed epistasis experiments with the adult fat body. Overexpression of a dominant-inhibitory form of dTOR (UAS-dTOR^{TED}) (24) both alone and in a background of activated insulin signaling did not affect whole-animal triglyceride levels, DNA content, or the triglyceride/protein, protein/DNA, or triglyceride/DNA ratios (Fig. 5A and B and 6A and B). In contrast, though expressing an active form of dFOXO, triple mutant dFOXO (dFOXO-TM), alone in the adult fat body had no effect on adult triglyceride levels, dFOXO-TM expression in a background of active insulin signaling suppressed the dInR^{A1325D}-induced triglyceride storage (Fig. 5C and D). In these experiments, we utilized a form of dFOXO rendered active by mutation of the three dAkt phosphorylation sites to alanine (27, 28, 40). To examine the requirement of sgg inhibition for the effects of insulin signaling on triglyceride storage, we overexpressed a constitutively active form of sgg. sgg is also regulated by wingless, raising the potential problem of distinguishing the effects of this pathway from those of insulin signaling. To avoid this ambiguity, we utilized a transgenic fly line that expresses a form of sgg in which the Akt phosphorylation site serine 9 has been mutated to alanine (sggS9A); this protein is resistant to the inhibitory effects of insulin signaling while normally responsive to wingless (5, 38). While overexpressing sggS9A alone in the adult fat body had no effect on triglyceride, in a background of activated insulin signaling, there was a trend for partial suppression of enhanced triglyceride storage that did not reach statistical significance (Fig. 5E and F). Thus, these data suggest that insulin signals to specific downstream targets to regulate fat storage.

As discussed above, the reduction in fat mass resultant from introducing constitutively active dFOXO or sgg could have been due to changes in cell number, triglyceride storage, or both. To resolve this issue, the fat body triglyceride content was normalized with respect to either the protein or the DNA content (Fig. 6). Both dFOXO-TM and sggS9A antagonized the increase in DNA, that is, cell number, generated by overexpression of dInR^{A1325D} in the adult fat body (Fig. 6C and E). However, FOXO-TM had no effect on the ratio of triglyceride to protein, triglyceride to DNA, or protein to DNA, while sggS9A substantially decreased the first two ratios (Fig. 6D and F). These data suggest that suppression of dFOXO acts downstream of dInR to regulate fat cell number exclusively, whereas inhibition of sgg is important for the control of both the cell number and the amount of triglyceride per cell.

DISCUSSION

In this study, we have shown that activating insulin signaling in the *Drosophila* fat body promotes triglyceride storage. These data contrast with those obtained when insulin signaling is decreased in the whole animal, during which triglyceride levels are elevated (4, 30, 52). However, the data in this study are consistent with the long-recognized role of insulin in vertebrate anabolic metabolism and the findings of previous studies using clonal overexpression of insulin-signaling pathway members in the *Drosophila* fat body (7). A number of possibilities may explain these differences. In insulin-deficient flies, extracellular sugar is increased, which may promote lipid storage by an insulin-independent mechanism (8, 43). It is also possible that the increased fat levels in flies in which insulin signaling is diminished may be secondary to the altered reproductive state of the animals. *C. elegans* nematodes with mutations in the insulin-signaling pathway enter a diapause state called dauer in which the worms decrease their reproductive fitness and increase their fat stores and life span (reviewed in references 6, 48, and 51). In *Drosophila*, some *dInR* and *chico* mutants also display decreased reproductive fitness and increased life span, raising the possibility that the modified fat content in these animals may be secondary to the other diapause-like phenotypes (4, 13, 52). Lastly, it is possible that altered insulin signaling in neurons may lead to secondary effects on nutrient balance by a mechanism analogous to that in rodents (39). In any case, the study results presented above demonstrate clearly

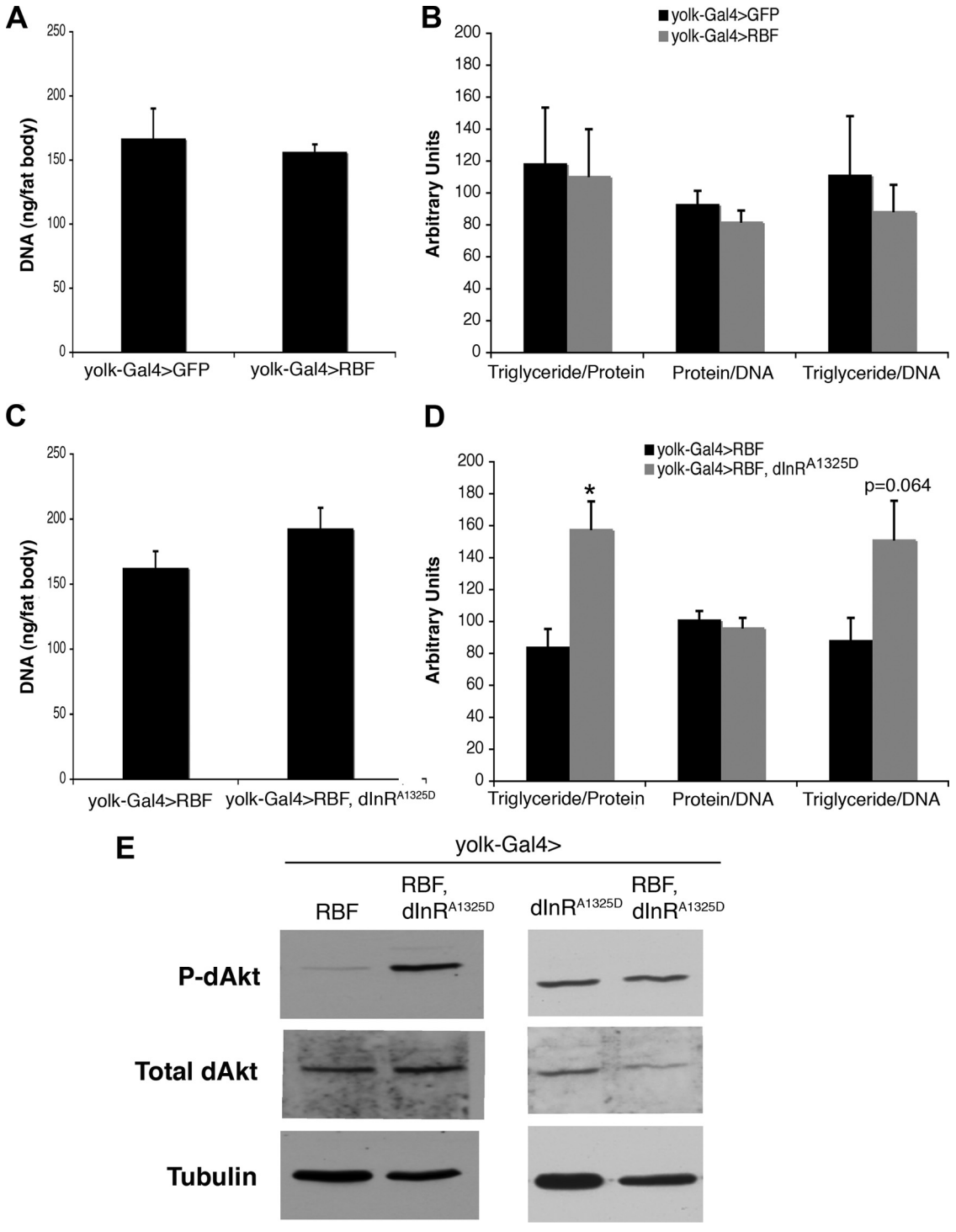

FIG. 4. Insulin regulates triglyceride storage independently of cell number. (A) Total DNA content of fat bodies dissected from 4- to 5-day-old adult yolk-Gal4>RBF virgin females or yolk-Gal4>GFP control animals. (B) Triglyceride/protein, protein/DNA, and triglyceride/DNA ratios in fat bodies dissected from yolk-Gal4>RBF 4- to 5-day-old adult virgin females or yolk-Gal4>GFP control animals. (C) Total DNA content of fat bodies dissected from 4- to 5-day-old adult yolk-Gal4>(RBF, dInR^{A1325D}) virgin females or yolk-Gal4>RBF control animals. (D) Triglyceride/protein, protein/DNA, and triglyceride/DNA ratios in fat bodies dissected from yolk-Gal4>(RBF, dInR^{A1325D}) 4- to 5-day-old adult virgin females or yolk-Gal4>RBF control animals. (E) Representative immunoblot of protein extracts made from fat bodies dissected from yolk-Gal4>RBF, dInR^{A1325D} 4- to 5-day-old virgin females or yolk-Gal4>RBF and yolk-Gal4>dInR^{A1325D} control animals. Each experiment was performed at least three times, and values are means ± SEM. *, $P < 0.05$; P-dAkt, phospho-dAkt.

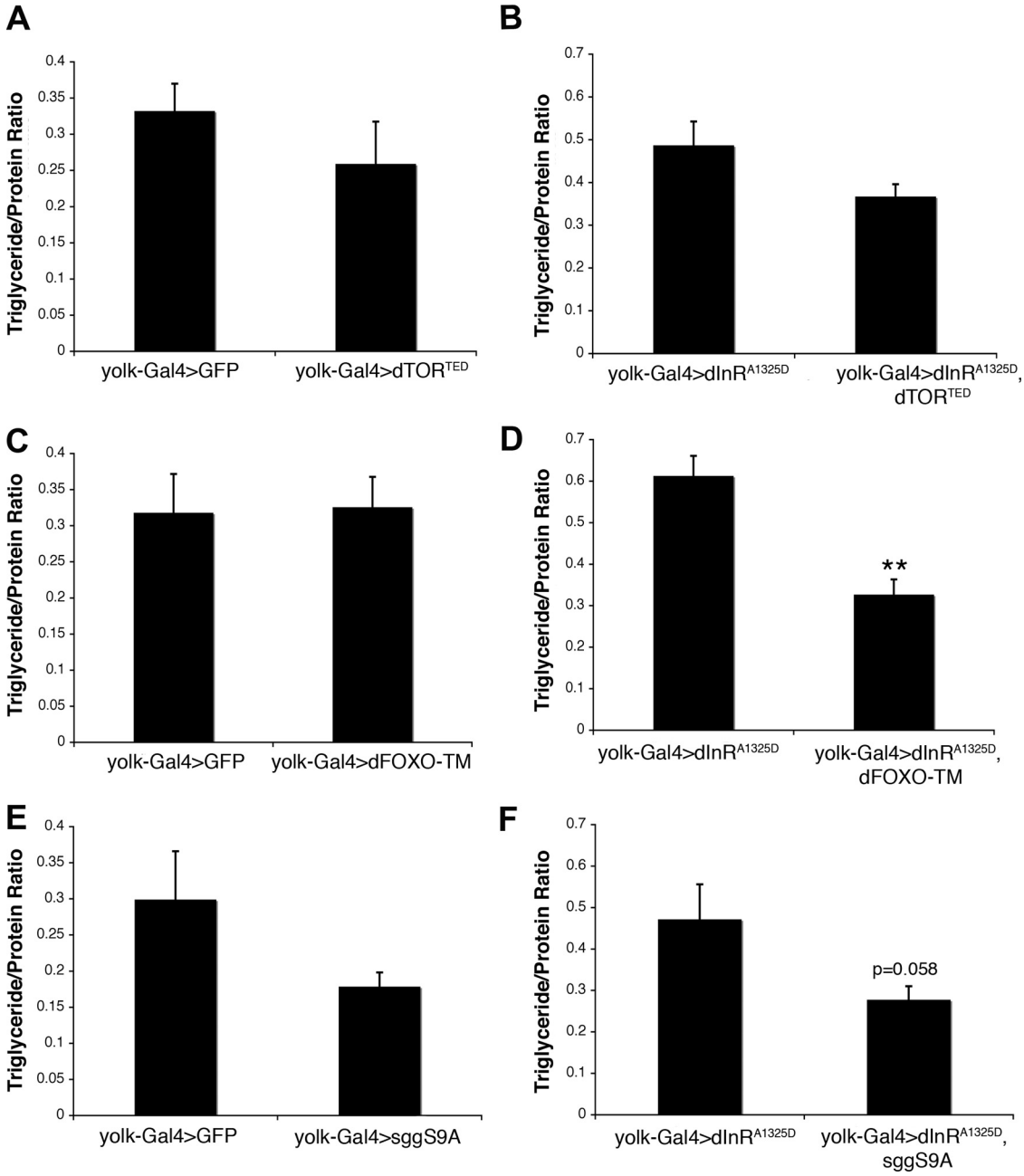

FIG. 5. Differential signaling downstream of dInR regulates triglyceride levels. (A) Triglyceride/protein ratios in individual yolk-Gal4>dTOR^{TED} 4- to 5-day-old virgin females compared to those in yolk-Gal4>GFP controls. (B) Triglyceride/protein ratios in individual yolk-Gal4>dInR^{A1325D}, dTOR^{TED} 4- to 5-day-old virgin females compared to those in yolk-Gal4>dInR^{A1325D} controls. (C) Triglyceride/protein ratios in individual yolk-Gal4>dFOXO-TM 4- to 5-day-old virgin females or yolk-Gal4>GFP controls. (D) Triglyceride/protein ratios in individual yolk-Gal4>dInR^{A1325D}, dFOXO-TM 4- to 5-day-old virgin females compared to those in yolk-Gal4>dInR^{A1325D} controls. (E) Triglyceride/protein ratios in individual yolk-Gal4>sggS9A 4- to 5-day-old virgin females compared to those in yolk-Gal4>GFP controls. (F) Triglyceride/protein ratios in individual yolk-Gal4>dInR^{A1325D}, sggS9A 4- to 5-day-old virgin females compared to those in yolk-Gal4>dInR^{A1325D} controls. Each experiment was performed at least three times, and values are means ± SEM. **, $P < 0.01$.

that in *Drosophila*, as in mammals, insulin signaling serves to promote fat storage in a cell-autonomous manner.

In the adult fat body, expression of an active insulin receptor promoted lipid storage through changes in both the fat cell number and the amount of lipid per cell. This pattern is reminiscent of the behavior of vertebrate preadipocyte cell lines, in which insulin or insulin-like growth factor 1 is critical for proliferation, differentiation, and storage of triglyceride following adipose conversion. In *Drosophila*, the adult fat body is distinct from the larval one, being derived from a separate developmental lineage (1, 26). Insulin signaling augments cell size and endoreplication in the larval fat body, but it is uncertain how

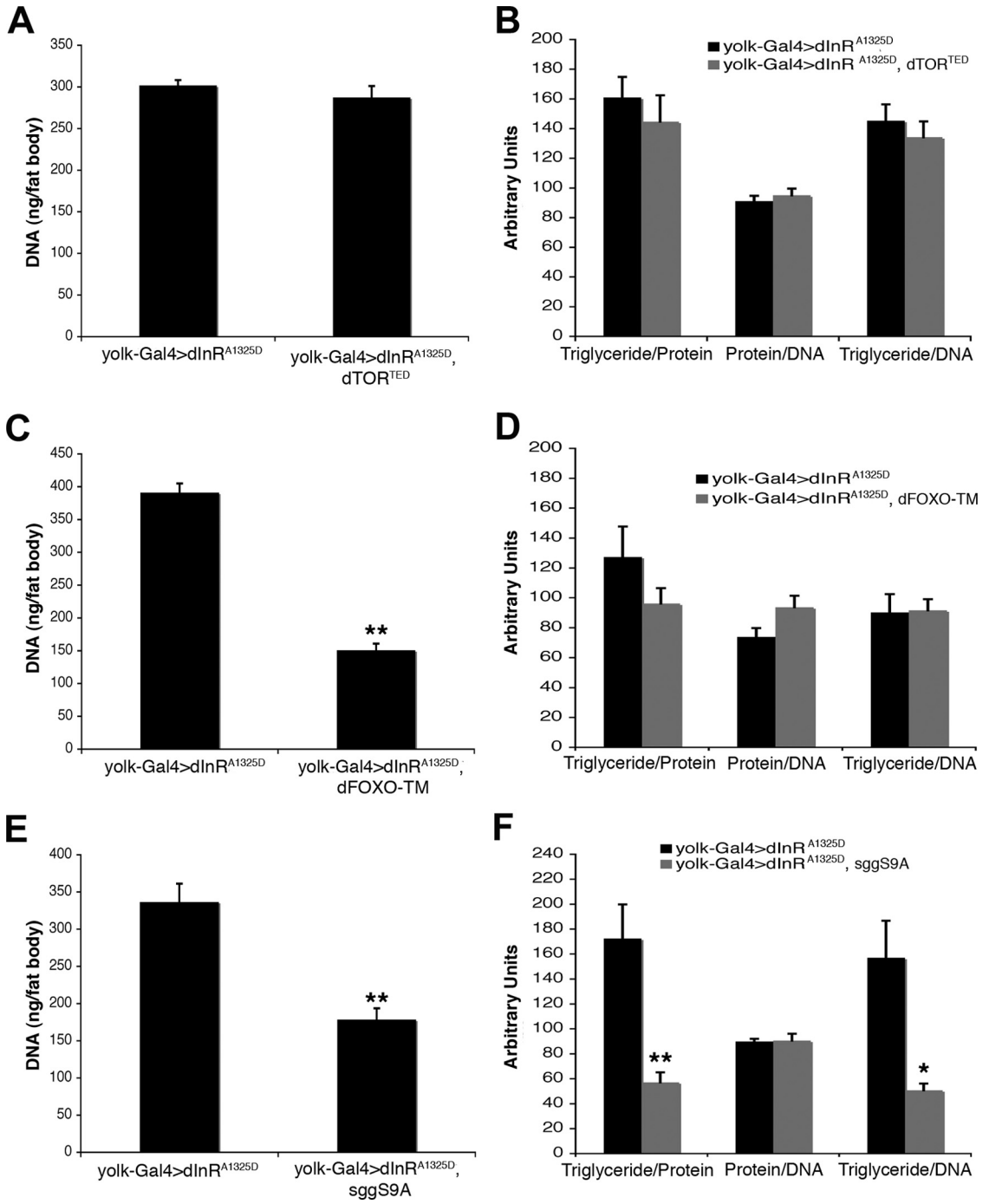

FIG. 6. dFOXO and sgg act downstream of dInR to regulate fat body cell number, while sgg acts downstream of dInR to regulate the amount of triglyceride per cell. Shown are the total DNA contents (A, C, and E) and triglyceride/protein, protein/DNA, and triglyceride/DNA ratios (B, D, and F) in fat bodies dissected from yolk-Gal4>dInR^{A1325D}, dTOR^{TED}, yolk-Gal4>dInR^{A1325D}, dFOXO-TM, and yolk-Gal4>dInR^{A1325D}, sggS9A 4- to 5-day-old adult virgin females, respectively, or yolk-Gal4>dInR^{A1325D} control animals. Each experiment was performed at least three times, and values are means ± SEM. *, $P < 0.05$; **, $P < 0.01$.

the same stimulus increases cell number in the adult (7). Proliferation of a committed precursor is a likely explanation, but as the nature of that cell in regard to the adult fat body is not known, a role for enhanced differentiation cannot be excluded. Manipulation of insulin signaling in other nonendoreplicating, mitotic cells of the larva leads to changes in cell number (11, 19, 56). In any case, the clearly discernible phenotype of increased fat cell number as well as triglyceride levels in response to expression of an active version of the insulin receptor allowed an assessment of downstream signaling by standard genetic strategies. Not surprisingly, abolishing the ability of insulin to promote dAkt-dependent phosphorylation and

inhibition of dFOXO in the adult fat body also eliminated the increase in cell number. In both mammals and *Drosophila*, FoxO1 resides downstream of and is inhibited by insulin signaling (2). In response to insulin, Akt phosphorylates FoxO1, causing it to be shuttled out of the nucleus and degraded (55). Among the many biological actions attributed to FoxO, positive regulation of proliferation, life span, and stress resistance takes place in both flies and mammals (2). For example, expression of dFOXO in the *Drosophila* eye or wing decreases organ size by affecting solely cell number and not cell size (28, 40). The role of suppression of FoxO as a mediator of the metabolic actions of insulin appears less clear. Its importance in the mammalian liver, in which inhibition of FoxO is a major means by which insulin represses gluconeogenic gene expression (23), has been most firmly established. The expression of both glucose 6-phosphatase and phosphoenolpyruvate carboxykinase genes is elevated during fasting, at least in part through the action of FoxO, and this effect is reversed by insulin-dependent phosphorylation catalyzed by Akt. The role of FoxO in hepatic lipid metabolism is more controversial (23, 31, 59). Manipulation of FoxO1 activity in the murine liver results in changes in triglyceride content, but it is unclear whether these changes are a direct effect or secondary to feedback modulation in insulin signaling. In contrast, there is a well-defined role for FoxO in cellular differentiation, including adipogenesis. The results of antagonizing transcriptional activation by FoxO1 explain a significant component of Akt's function in supporting adipocyte differentiation (35). However, even in this situation, the requirement for FoxO may well be in mitotic expansion, i.e., the several rounds of division that preadipocytes undergo prior to differentiation (35, 58). These observations are consistent with the data presented herein that dFOXO-TM antagonizes the insulin-dependent increase in fat body cell number, emphasizing the conservation of this pathway in the regulation of adipose mass.

While the results of the experiments described herein indicate a role for dFOXO in regulating cell number and not the amount of fat per cell, a previous study has documented that dFOXO-TM overexpression in the *Drosophila* head fat body leads to enlarged lipid droplets (27). While the same active dFOXO (dFOXO-TM) transgene was used in both studies, this discrepancy may be explained by the difference in the methodologies employed. Hwangbo et al. utilized histological methods to measure lipid droplet size, whereas the study described herein relied on biochemical measurements of fat body triglycerides (27). It is possible that the larger lipid droplets observed in dFOXO-TM-expressing fat bodies in the previous study (27) may not correlate with a net gain of triglyceride, providing an explanation for the different results observed here by measuring triglycerides directly. Different Gal4 drivers were also used in these two studies, and their different temporal and spatial control of dFOXO-TM expression may be responsible for the differing results.

The absence of effect of dominant-inhibitory dTOR on lipid accumulation downstream of insulin is somewhat surprising, given the suggestion that the TOR pathway is important to lipid metabolism. In addition to regulating cell and organ growth in *Drosophila*, a role for the TOR pathway in metabolism has been appreciated recently. A hypomorphic mutation of dTOR results in reduced fat body triglyceride levels, with a concomitant decrease in the expression of the lipogenic *Drosophila* fatty acid synthase gene and an increase in the expression of the brummer lipase gene (30). The mutants also have decreased hemolymph sugar levels, suggesting a glucose-regulatory role for the TOR pathway in addition to the control of lipid storage (30). Evidence implicating the targets of TOR, S6K and 4E-BP, in regulating energy homeostasis also exists. *Drosophila* 4E-BP (d4E-BP) mutants are starvation sensitive due to decreased lipid levels; conversely, overexpression of an active form of d4E-BP increases whole-animal triglyceride levels (53). This role of d4E-BP in regulating lipid storage is consistent with that observed in mammals (54). Thus, although TOR signaling has been shown to regulate lipid storage, the signal activating TOR in this process is unknown. Given the findings presented above indicating a lack of effect of dominant-inhibitory TOR on insulin-dependent triglyceride accumulation, it is likely that TOR in flies regulates lipids in response to a stimulus such as amino acids rather than insulin.

In contrast to the profound effect of active dFOXO on cell number in the fat body, the ability of nonphosphorylatable sgg to suppress the insulin-dependent increase in triglyceride content was completely unanticipated. GSK3 is a signaling intermediate in at least two pathways, the Wnt/wingless and the insulin/PI3K pathways, although phosphorylation by Akt is important only to the latter (34). The role of GSK3 in regulating mammalian adipocyte differentiation is related to Wnt signaling and thus is not relevant to the effects on lipid accumulation found in this study (29, 42). A genetic deficiency in GSK3 partially suppresses diabetes in animal models, but this finding has not been linked to alterations in lipid metabolism (49). A function for GSK3 as a determinant of lipid storage levels in either the mammalian adipocyte or the mammalian hepatocyte has not been ruled out, and the strong conservation of insulin-signaling pathways between *Drosophila* and higher vertebrates suggests that closer examination of this possibility is warranted. Interestingly, GSK3 phosphorylates SREBPs, causing recruitment of the ubiquitin ligase SCF^{Fbw7} and targeting for ubiquitination and degradation by the proteosome (47). This phenomenon has been studied in regard to its role in the modulation of lipogenesis occurring with the cell cycle but has not been evaluated for its relevance to metabolic signaling by insulin, nor has it been tested genetically in liver.

In summary, this study demonstrates that insulin promotes triglyceride storage in the *Drosophila* fat body by regulating both cell number and lipid accumulation. Moreover, the complete effect of insulin depends upon the inhibition of signaling through both dFOXO and sgg, raising the novel possibility that the latter is a key signaling intermediate in insulin's critical role in anabolic lipid metabolism.

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